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GRANT NUMBER DAMD17-94-J-4001

TITLE: The Role of Alveolar Macrophages and Chemical Mediators in a Model of Smoke-Induced Lung Injury

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REPORT DATE: December 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1996	Final (22 Nov 93 - 21 Nov 96)

4. TITLE AND SUBTITLE
The Role of Alveolar Macrophages and Chemical Mediators in a Model of Smoke-Induced Lung Injury

5. FUNDING NUMBERS
DAMD17-94-J-4001

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

Using a rabbit model of lung injury to simulate fire-related transport accidents and military combat situations, our studies have demonstrated that toxic products of combustion cause a severe inflammatory reaction in the lung parenchyma, as evidenced by gas exchange, WtL/WtB, O_2^- and TNF -α by PAM *in vitro*, and pathological evidence. It was suggested that pulmonary alveolar macrophages play an important role in the early phase of acute lung injury through production of oxygen radicals and cytokines. Standard doses of U75412E (1% in saline) were aerosolized and ventilated into the rabbit lungs via an endotracheal tube either before or after smoke exposure, suggesting the treatment with U75412E significantly prevented or limited the extent of acute lung injury due to smoke insult. It is postulated that lazaroids may possibly be associated with the oxygen radicals-initiated processes which activate cytokine gene transcription and initiate the cytokine cascade as a result of the smoke insult. Also our findings have demonstrated that this lazaroid may be more effective as a rescue agent rather than a prophylactic agent for acute smoke-induced lung injury. The additional benefit from dose- and time-effects of U75412E remain to be experimentally demonstrated.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Smoke, Alveolar Macrophages, Superoxide Radical, Surfactant, Lazaroids	27
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Mark L. Fitter 12-17-96

PI - Signature Date

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INTRODUCTION

Smoke-induced acute lung injuries (SI-ALI) remain one of the most severe and lethal complications associated with fire-related disasters in an enclosed space [1]. An immediate catastrophic pulmonary failure and the subsequent development of pneumonia produce substantial morbidity and mortality. Each year in the United States approximately two million people are injured in fires, of which 130,000 are hospitalized, and more than 50% require intensive care. The majority of deaths, 8000 to 12,000 annually, are caused by the toxic productions of combustion [2]. Furthermore, the presence of inhalation injury can increase the risk of dying by a factor of five in burn victims.

SI-ALI are usually produced by a variety of toxic gaseous or particulate products of incomplete combustion. The combination of burning material and an enclosed space are major factors that lead to SI-ALI in transportation accidents and other catastrophic scenarios. Direct thermal injuries to lower airways are extremely rare because of rapid dissipation of heat through air passages, except in those patients subjected to the effects of hot gases or high-pressure steam from sudden explosions or similar accidents [3,4]. Adult respiratory distress syndrome (ARDS) is an important consequence of smoke inhalation. The primary pathophysiological abnormality of SI-ALI is an injury to the alveolar-capillary barrier resulting in increased lung permeability. There are rapid and massive accumulations of fluid in the interstitial and alveolar spaces of the lung accompanied by hemorrhage. There are four stages in the clinical course of patients with smoke-inhalation lung injury: asphyxia and acute poisoning, acute respiratory insufficiency, bronchopneumonia, and recovery. Most inhalation injury-related deaths occur during the second and third stages, which is involved in alterations in local and systemic defense mechanisms. Patients that survive these complications may enter the recovery stage [5]. Therefore, knowledge about the early role of alveolar cells in the pathogenesis of smoke-induced acute lung injury should also benefit the management of burn victims with smoke insult.

SI-ALI are characterized by a complex series of events in the alveolar septal area, such as the cascade of proinflammatory cytokines, the propagation of toxic oxygen reactive species and the release of inflammatory mediators. Of these mediators, the role of oxygen reactive species, especially lipid peroxides, is thought to be one of major pathological manifestations of lung alveolar injury [6-8]. There is strong evidence that lipid peroxides are directly toxic to alveolar cells [9,10], which results in an increase in capillary permeability and lung edema. Meanwhile, oxygen-derived free radicals are rapidly capable of activating cytokine gene transcription and initiate the cytokine cascade as a result of the smoke insult, including that for TNF-α [5]. Most importantly, pulmonary alveolar macrophages (PAM) could be activated and/or altered both by smoke combustion products, such as nitrogen dioxide, ozone and particulates, and free radicals from activated leukocytes [11,12].

It would be of interest to explore the potential of a compound in protection of SI-ALI in which oxidation damage has been implicated, either by rescue or by prophylactic treatment. A novel class of antioxidants, 21-aminosteroids (Lazaroids), have been extensively investigated and demonstrated to possess therapeutic potential in acute central nervous system trauma, cerebral ischemia and subarachnoid hemorrhage [18-21], and to serve a protective role in heme protein-induced renal injury and splanchnic artery occlusion shock [22]. The mechanism known so far to explain the action of lazaroids is their strong lipid antioxidant activity attributed to iron chelation, chain breaking like that of α-tocopherol, and possibly the alteration of membrane fluidity [22-25]. In particular, it has recently been suggested that lazaroids also possess therapeutic properties in animal models of endotoxin [26], bleomycin [27], *Escherichia coli* [28], silica [29], hyperoxia [9], and ischemia-reperfusion [30,31] induced lung injuries. However, very little information is known about the pharmacological effects of lazaroids on acute smoke-induced lung injuries, which involves a complicated pathogenesis ultimately leading to ARDS [32].

It has been documented that the early events of smoke-induced lung injury will eventually lead to serious outcomes including death within several hours [32]. The initiation of acute lung injury is associated with activation of pulmonary alveolar macrophages (PAM) in the distal airspace. There are several studies [33-36] which demonstrate that PAM tumor necrosis factor-α (TNF-α) and superoxide anion (O_2^-) may be the chemical mediators released early in the lung injury process and may play a pivotal role in the pathogenesis of acute lung injury. There is evidence to suggest that the alveolar macrophage-epithelial cell axis may be important for the initiation and evolution of acute lung injury [37,38]. Therefore, PAM lavaged from rabbits exposed to *in vivo* 60 tidal volume breaths of diesel fuel-polycarbonate plastic smoke with U75412E treatment, either rescue or prophylactic mode, were analyzed for alterations in their ability to produce superoxide anion and TNF-α *in vitro* after cell isolation and culture. The purpose of the study was to investigate the potential value of the 21-aminosteroid analog U75412E for treatment of acute smoke-induced lung injuries and the lazaroid's ability to modulate the effect of oxygen radicals in a rescue mode of therapy. Our hypothesis in this study is that U75412E treatment attenuates the smoke-induced lung injury process by altering the release of chemical mediators by PAM.

MATERIALS AND METHODS

Animals

A total of 242, specific pathogen-free, female New Zealand white rabbits, weighing 2.33-3.22 kg, were utilized in this study which was approved by the University of Arizona Animal Care and Use Committee. The animals were assigned to the following exposure groups: (1) sham smoke controls (CON); (2) U75412E treatment before sham smoke control (DFC); (3) U75412E treatment after sham smoke control (DLC); (4) smoke exposure (SMO); (5) U75412E treatment before smoke (D+S); (6) U75412E treatment after smoke (S+D). Each group of rabbits were divided into both one and two hour subgroups, which were again divided for either bronchoalveolar lavage fluid (BALF) or pathological studies. The rabbits were housed one per cage with a 12-hour light/dark cycle at the Arizona Health Sciences Center AAALAC-approved animal resource facility and fed a standard rabbit chow diet and tap water *ad libitum.*

Surgery

The rabbits were anesthetized intramuscularly with a mixture of ketamine HCL (50 mg/kg; Parke-Davis, Morris Plains, NJ, U.S.A.), xylazine (8 mg/kg; Mobay, Shawnee, KS, U.S.A.), and acepromazine maleate (1 mg/kg; Fermenta, Kansas City, MO, U.S.A.) for the entire experimental period to ensure a deep state of anesthesia. A tracheostomy was performed with an endotracheal tube (ET, Concord/Portex, Keene, NH, U.S.A.) firmly tied in place with its tip 2 cm above the carina. The rabbits were then paralyzed with 8 mg/kg of intravenous gallamine triethiodide (Flaxedil; Lederle, Carolina, Puerto Rico, U.S.A.) to suppress spontaneous respiration during the entire experimental process. Auricular arterial blood was taken 5 minutes before the end of the experimental time period and analyzed using a System 1620 pH/Blood Gas Analyzer and 482 Co-Oximeter (Instrumentation Laboratory, Lexington, MA, U.S.A.). At the end of the experimental time period, the rabbits were killed by exsanguination of the abdominal aorta, and the heart-lung block was removed immediately for bronchoalveolar lavage (BAL) or pathological studies.

Smoke exposure

The smoke exposure protocol has been described in detail in a previous study [39]. Briefly, polycarbonate plastic shavings (0.2 g) and 20 ml diesel fuel were placed in a ceramic

crucible, and set on fire in a stainless steel smoke chamber. A total of 60 tidal volume breaths, based on a calculation of tidal volume at 14 ml/kg body weight, of diesel fuel-polycarbonate plastic smoke was drawn from the sampling port with a 60 cc syringe and then injected into the rabbit's lungs via the ET, while the ventilator was interrupted. Sham smoke exposure rabbits had the same protocol as smoke exposure except that ambient air was drawn through an empty smoke chamber. A piston-type ventilator (Model 665, Harvard Apparatus Co., South Natick, MA, U.S.A.) was used to deliver a tidal volume of 14 ml/kg at a rate of 50 breaths/min. to maintain $PaCO_2$ at 35-45 mmHg.

Drug treatment

The lazaroid U75412E (21-[4-(3-ethylamino-2-pyridinyl)-1-piperazinyl]-16-α-methylpregna-1,4,9-(11)-triene-3,20-dione), manufactured by Pharmacia-Upjohn Inc. (Kalamazoo, MI, U.S.A.), was examined in this study. We aerosolized 0.0418 g of sterile U75412E and 0.125 g encapsin solution in 4.18 ml of normal sterile saline (1% U75412E solution) for 3 minutes using a DeVilbiss Pulmo Sonic nebulizer (Model 25, Somerset, PA, U.S.A.). The nebulizer circuit was temporarily placed into the ventilator circuit and aerosolized lazaroid was ventilated into rabbit lungs through the ET either immediately before smoke exposure or at 0.5 and/or 1.5 hours after smoke exposure.

Isolation and culture of pulmonary alveolar macrophages

PAM were harvested by a revision of the Myrvik *et al.* [40] method of bronchoalveolar lavage. Washes were repeated four times with 60-ml aliquots of sterile 0.85% saline solution flushed through the tube portion of the ET with 90.2% ± 4.2 mean recovery. The lavage fluid was decanted into 20-ml chilled polypropylene tubes and spun at 500x*g* for 5 min. at 4 °C. The supernatant was used for determination of total protein in BALF, through a modification of the Lowry micro method using diagnostic kits (Sigma, St. Louis, MO, U.S.A.). The cell pellet was frozen at -75 °C without preservatives and saved for future analysis. A 50-ml centrifuge tube containing BALF was centrifuged for 15 minutes at 500x*g*. The supernatant was decanted and saved in sterile microfuge tubes and stored at -70 °C for BALF TNF-α assays. All cell pellets were resuspended in RPMI 1640 media (GIBCO Laboratory) with L-glutamine and sodium bicarbonate, combined, and centrifuged again at 500x*g* for 15 min. Cell counts showed average yields of 35-70 million cells per rabbit with 95% viability (trypan blue exclusion). An aliquot of 200 µl of cell suspension (1x 10^6 PAMs/ml) was added to 2 ml of RPMI 1640 medium with 25 mM HEPES in Costar 35 mm culture plates and the PAM were allowed to adhere for 30 min. at 37 °C, 5% CO_2. Media was then decanted and replaced with RPMI 1640 supplemented with 25 mM HEPES containing 10% heat-inactivated (56 °C, 30 min.) fetal bovine serum (Gemini, Calabasas, CA, U.S.A.) and 1% penicillin/streptomycin. Lipopolysaccharide (LPS, 1 µg/ml) was added to some cultures at this time. Cells remained in culture for either 2 or 24 hours. After culture, supernatants were saved at -70 °C for TNF-α assay and cells were measured for superoxide production.

TNF-α bioassay

The TNF-α bioassay [41,42], utilizing a WEHI-164 cell line subclone 13 (Dr. Waage's Laboratory, Institution of Cancer Research, The University of Trondheim, Trondheim, Norway), was performed in the study. The cell numbers of the WEHI-164 cell line subclone 13 were adjusted to 5×10^5 cells/ml in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 30 µg/ml of gentamicin (Sigma, St. Louis, MO, U.S.A.), and 0.5 µg/ml of actinomycin D. A 100 µl aliquot of BAL or cell culture supernatant were run in duplicate or triplicate and serial dilution performed in a plate (flat-bottom 96 wells, Corning 25860) mixed with 100 µl of the above

mentioned WEHI-164 subclone 13 cell suspension. The plates were then incubated at 37° C, 5% CO_2 for 24 hours. After incubation, a 20 µl aliquot of [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) at a concentration of 5 mg/ml in 0.01 M phosphate buffered saline (PBS) was added to each well for 4 hours at 37° C, 5% CO_2. The supernatant was discarded, and 100 µl of isopropanal-HCl (0.04N) was added to each well. The plates were kept in the dark at room temperature overnight, and then read at a test wavelength of 570 nm and reference wavelength of 660 nm on a MR600 microplate reader. The murine rTNF-α (10 ng/µl, Genzyme Corporation, Cambridge, MA, U.S.A.) was used as the standard for this assay in a range of 0.2 to 200 pg/ml. Polyclonal antiserum (anti-murine and anti-human TNF-α, Genzyme Corporation, Cambridge, MA, U.S.A.) and monoclonal antibody (anti-murine TNF-α from Genzyme; antihuman TNF-α from Olympus, Lake Success, NY, U.S.A.) were used to determine the cytotoxic specificity of TNF.

PAM superoxide bioassay

Pulmonary alveolar macrophages from the lavage were used to determine oxygen radical production by the single-cell measurement method [43]. This technique uses electro-optical density measurement methods to determine reduction of nitroblue tetrazolium (NBT), as an indicator of oxygen radical production. After incubation, supernatants were discarded, and 3 ml of resuspension solution [140 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, and 10 mM Na-HEPES (N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid)] containing 2 mg/ml of NBT were added. Plates were then transferred to a 37 °C warm stage of an inverted microscope and a layer of paraffin oil was placed over the solution to prevent evaporation. Cells were transilluminated at 550 nm, the peak absorbance wavelength for insoluble diformazan in aqueous solutions. A field of six PAM was randomly selected, viewed with a low light level silicon-vidicon television camera, and video-recorded for 55 minutes using a 3/4 inch Sony (Model V05600) videocassette recorder. PAM were stimulated to produce superoxide (O_2^-) by the addition of 10 µg/ml opsonized zymosan, 25 minutes after being video-recorded. Temporal changes in light intensity over PAMs were determined by playing back previously recorded images through a digital image analysis system capable of measuring the optical density of specified areas of the video image. Background light intensity variation was determined in regions of the image containing no cells. A modifed form of the Beer-Lambert equation relating concentration to optical density was used to calculate the mass of formazan produced as a result of the reduction of soluble nitroblue tetrazolium dye to insoluble diformazan precipitate. Oxygen radical production was calculated assuming a two-electron transfer for full reduction of nitroblue tetrazolium to formazan. Data were expressed as superoxide production (fmole/cell).

Stable Metabolite of PGI_2 (6-keto-$PGF_{1\alpha}$ Assay)

The collected fluid for 6-keto-PGF1alpha analysis was decanted into chilled polypropylene tubes and spun at 1500 rpm for 5 min at 4 °C. The supernatant was decanted from the cell pellet and frozen immediately at -75 °C for analysis. We determined BALF 6-keto-$PGF_{1\alpha}$ concentrations by radioimmunoassay techniques as described elsewhere (39) using standard kits (DuPont NEN Research Products, Inc., Boston, MA). The 6-keto-$PGF_{1\alpha}$ antibody has the highest cross-reactivity (0.8%) with prostaglandin E_1. All samples were run in duplicate and averaged to obtain a mean value.

Pathological evaluation

After removing the heart-lung bloc, the esophagus and cardiovascular structures were carefully dissected away from the body and the tracheopulmonary bloc weighed. The lungs were

fixed with 1/2 strength Karnovasky's fixative (2% paraformaldehyde, 2% gluteraldehyde, and 0.01% picric acid in 0.1 molar HEPES) at 20 centimeters H_2O pressure [44] and room temperature for one hour. The lungs were then immersed in fixative for 24 hours at 4 °C. Sections (2-3 mm in thickness) from the fixed lungs were taken from the mid-portion of the left and the right inferior lobes for light microscopy and minced into 1 mm^3 pieces for electron microscopy. Light microscopy sections (5 μm) were embedded in paraffin and stained with hematoxylin and eosin (H & E). Electron microscopy sections (silver to gold interference colors) were osmicated and stained with lead citrate and uranyl acetate. The electron microscopy sections were examined with a Philips CM-12 electron microscope. Both light and electron microscopy slides were examined using blinded techniques.

Statistical analysis

Mean (SEM) data were calculated for each group of rabbits or cells. Analysis of variance (ANOVA) statistical analyses were calculated using the Statview (Abacus Concepts, Inc., Berkeley, CA, U.S.A.) computer program. A priori contrast between groups was performed using the Sche'ffe F test. Comparisons with $p < 0.05$ were considered significant.

RESULTS

Lung gas exchange

The SMO groups for either one or two hour experimental protocols had a significant decrease in blood PaO_2 and pH values accompanied by a marked increase in blood $PaCO_2$ when compared to the CON group (Figures 1 and 2). However, the U75412E-treated groups, including the D+S and S+D groups, had a significant elevation of blood PaO_2 and pH values, and a significant attenuation of $PaCO_2$ compared to the SMO group. However, the changes in these blood parameters did return to control levels in S+D2 group. There were no differences in gas exchange values between the CON and DFC groups.

Cell count, differential in BALF

In the one hour post-smoke experiment time-period, the BALF cell count in the SMO group was considerably higher than that observed in the CON group. Conversely, a significant attenuation of BALF cells in the U75412E pretreatment group (D+S) was observed when compared to the SMO group. However, this BALF cell count was still elevated when compared to the CON group (Table 1). The percentage of PAM in the SMO group was lower than the other groups, but only reached a significant decrease compared to that of the U75412E pretreatment group. The percentage of granulocytes in the SMO group was higher than the CON group, but the U75412E pretreatment groups had a significant decrease compared to that of the SMO group. In the two hour experiment time-period, the BALF cell count in all smoke groups had a decrease compared to that of the SMO group, but only D+S group and its control DFC group reached statistical significance. No statistical difference was demonstrated in lymphocyte differential (%) among all experimental groups.

Superoxide anion (O_2^-)

In the one hour post-smoke experiment time-period, or at 2 hours after cell isolation and culture, both background and zymosan-stimulated production of PAM superoxide anion (O_2^-) in the SMO1 group were significantly decreased compared to that of the CON1 group (Figure 3). The production of PAM O_2^- in both U75412E treatment groups (D+S1, S+D1), either background or zymosan-stimulated group, followed the same pattern as that in the SMO1 group, but background values of the S+D1 group only reached statistical significance when compared to

its corresponding control (CON1). Conversely, at 24 hours after culture, both background and zymosan-stimulated production of PAM O_2^- were significantly increased in the SMO1 group as compared to that of the CON1 group. However, both background and zymosan-stimulated PAM O_2^- values with or without LPS stimulation were significantly attenuated in the S+D1 group, not in the D+S1 group, as compared to that of the SMO1 group. There were no significant differences in PAM production of O_2^- at 24 hour between groups with and without LPS-stimulation.

In the two hour experiment time-period, background production of PAM superoxide (O_2^-) in the SMO2 group (4.49 ± 1.44 fmole/cell, N=9) was significantly increased compared to that of the CON2 group (0.81 ± 1.29 fmole/cell, N=9). The PAM O_2^- level in the D+S2 group (4.92 ± 1.71 fmole/cell, N=9) was identical to that in the SMO2 group, but the PAM O_2^- value of the S+D2 group (0.73 ± 0.56 fmole/cell, N=6) decreased compared to its corresponding control level. Zymosan-stimulated production of PAM O_2^- had the same trend as that of background production of PAM O_2^-, but there were no significant differences in PAM production of O_2^- among all experimental groups.

Tumor necrosis factor-α (TNF-α)

After cessation of the experimental protocol at one hour, BALF TNF-α levels in the SMO1 group appeared to increase, but did not reach statistical significance when compared to that of the CON1 group. BALF TNF-α levels in the D+S1 group had a significant decrease compared to that of the DFC1 group (Table 2). At 24 hours after cell isolation and culture, PAM TNF-α levels in the SMO1 group were 3.6-fold greater than in the CON1 group. PAM TNF-α levels in both U75412E pretreatment groups significantly decreased when compared to those in the SMO1 group, however, the levels did not return to CON1 values. There were no significant differences in PAM TNF-α levels at 24 hours after stimulation with LPS among all experimental groups.

At two hours post-smoke, BAL TNF-α levels in the SMO2 group (2.0 ± 0.4 pg/ml) and the D+S2 group (7.5 ± 2.3 pg/ml) were significantly increased when compared to that of control (0.8 ± 0.3 pg/ml), the S+D2 group TNF-α value did not have a significant decrease when compared to that of the SMO2 group (1.5 ± 0.3 pg/ml, $p>0.05$). PAM TNF-α levels in the SMO2 group were significantly higher than in the CON2 group, either without or with LPS-stimulation. However, PAM TNF-α levels in both D+S2 and S+D2 groups significantly decreased (except S+D2 with LPS-stimulation) when compared to those in the SMO2 group, regardless of LPS stimulation. There were differences of PAM TNF-α levels between D+S2 and S+D2 groups, but these values did not reach statistical significance.

PGI_2, measured as its stable metabolite, 6-keto-$PGF_{1\alpha}$

The SMO1 group (24.42±2.01 pg/ml) caused significant increases in bronchoalveolar lavage concentrations of 6-keto-$PGF_{1\alpha}$, which had a 1.79-fold increase compared to the CON1 group (12.37±3.72 pg/ml). There were no differences in the bronchoalveolar lavage concentrations of 6-keto-$PGF_{1\alpha}$ between SMO and U75412E treatment groups, either before or after smoke insult.

Histopathological analysis

Gross pathology showed that rabbit lungs in the SMO group had a dark red appearance. Wet lung/body weight ratio (WtL/WtB) in the SMO group was significantly increased compared to that of the CON group either at one or two hours experimental protocol (Figure 4). However, the WtL/WtB in both U75412E treatment groups (D+S, S+D) were considerably attenuated when compared to that of the corresponding SMO group values. In all the rabbit lungs in which smoke was instilled, minute soot particles were observed in the alveolar spaces and within PAM. Scattered minute inflammatory foci were observed in 50% of the lungs of SMO rabbits, but these

foci did not appear to be related to the accumulation of soot particles in the lungs. Electron microscopy also revealed that a significant attenuation of interstitial edema in the alveolar walls and the vacuolization of the type II cells occurred in the U75412E treatment groups, when compared to the SMO group. Additionally, we measured epithelial thickness and quantified sloughing of epithelium to the basement membrane in H&E stained rabbit terminal bronchioles. Epithelial thickness and sloughing did not significantly change in all smoke exposure groups at the 1 and 2 hour experiments as compared to control. Interestingly, an insignificant increase in epithelial thickness, as compared to control, occurred in the 1 hour experiment in the U75412E treated group.

DISCUSSION

Using a rabbit model of lung injury to simulate fire-related transport accidents and military combat situations, our studies have demonstrated that toxic products of combustion cause a severe inflammatory reaction in the lung parenchyma [32,45-54], as evidenced by gas exchange, WtL/WtB, O_2^-, and TNF-α by PAM *in vitro*, and pathological evidence. However, standard doses of U75412E (1% in saline) were aerosolized and ventilated into the rabbit lungs via an endotracheal tube either before or after smoke exposure, suggesting the treatment with U75412E significantly prevented or limited the extent of acute lung injury due to smoke insult [46-48]. It was also demonstrated that this lazaroid may be more effective as a rescue agent rather than a prophylactic agent for acute smoke-induced lung injury [48]. The additional benefit from larger and/or multiple doses of U75412E remain to be experimentally demonstrated.

The role of alveolar macrophages and chemical mediators, as well as the effects of lazaroid on an acute smoke-induced lung injury

The present study demonstrated that acute smoke exposure markedly decreased blood PaO_2 and pH values while increasing $PaCO_2$. However, a single aerosolized U75412E dose (1% solution) for 3 minutes resulted in a significant improvement in the gas exchange defect generated by the smoke insult, while with double doses treatment these values did return to control levels. We disclosed a protective role of U75412E on gas exchange in this smoke model similar to that of U74389G, another lazaroid compound, demonstrated by Johnson and co-workers in a lung injury dog model [55]. Pretreatment with U74389G (10 mg/kg body weight) attenuated the decrease in PaO_2 (476 ± 61 mmHg to 226 ± 143) and the increase in intrapulmonary shunt ($12.6\% \pm 6.1\%$ to $14.3\% \pm 6.8\%$) 70 minutes after endotoxin infusion.

Alveolar macrophages are the major resident phagocyte on the air-exchange surface of the lung and serve as the primary cellular defense mechanism of the lung. There is evidence to indicate that the alveolar macrophage-epithelial cell axis may be important for the initiation and evolution of acute injury [37]. We found that the smoke insult resulted in an increase in the total cell count by 88.9% and additional recruitment of granulocytes into the lung airspace within the one hour experiment time-period compared to controls. The present data suggests that the lung phagocytic system may play an early and possibly crucial role in the inflammatory processes by taxis and phagocytosis of large amounts of smoke particulate matter. Conversely, a single aerosolized U75412E dose (1% solution) for 3 minutes prior to smoke exposure attenuated the elevation of the total cell count and granulocytes in BALF. We postulate that this effect may involve either the attenuation of chemoattractants produced at the smoke-induced inflammatory focus or the alteration of chemotaxis characteristics of blood monocytes and interstitial macrophages [33,34].

Alveolar macrophages may contribute to lung injury and inflammation in multiple ways, including release of reactive oxygen species such as superoxide anion [56]. Phagocytosis is usually accompanied by a respiratory burst that increases cellular oxygen consumption and glucose metabolism in the hexose monophosphate shunt which leads to the release of highly reactive oxygen metabolites at the surface of plasma membranes [43]. The initial oxygen metabolite is thought to be superoxide anion, which can readily form hydrogen peroxide, hydroxyl ion, singlet oxygen, or hydrochlorous acid. In order to determine the effects of treatment with U75412E on PAM function following intratracheal smoke instillation, PAM lavaged from rabbits were analyzed for alterations in their ability to produce superoxide anion *in vitro*. The data showed that acute smoke exposure *in vivo* activated and enhanced the PAM O_2^- production *in vitro*. It is possible to build a cause-effect relationship of the lung injury based on this study that smoke exposure significantly altered the function of PAM. Also a biphasic response of production of PAM O_2^- regardless of the presence of opsonized zymosan was observed in the SMO group. At 2 hours after cell isolation and culture, production of PAM O_2^- was significantly inhibited, while at 24 hours, production of PAM O_2^- was significantly increased when compared to the corresponding control (CON). This may be one of the characteristics of PAM associated with smoke exposure due to an immediate release of O_2^- during the smoke insult. However, the U75412E treatment at 0.5 hour post-smoke clearly interrupts the long-term PAM O_2^- production, suggesting that treatment with U75412E may be more effective if administered in a "rescue" mode after the smoke insults.

Previous studies suggest that TNF-α may be an early predictive marker and potent proinflammatory cytokine in inducing acute lung processes [36]. The data showed that acute smoke exposure *in vivo* activated and enhanced PAM TNF-α release *in vitro*. Therefore, the release of TNF-α from PAM and its presence in the alveolar septal area may be an important factor that induces acute lung injury and inflammation [38]. We demonstrated that the U75412E treatment, either before or after smoke insult, significantly attenuated the elevation of smoke-induced PAM TNF-α production *in vitro* even if the TNF-α values did not return to control levels. Perhaps, the higher dose of lazaroid affects the ability of lung cells, both resident and recently recruited, to produce TNF-α. Thus, the modulation of PAM TNF-α production may possibly be one of the pharmacological action mechanisms to attenuate lung injury in this model. However, the dose- and time-effect relationships should be built to confirm the action of U75412E to attenuate PAM TNF-α production.

Histopathological data showed that treatment with U75412E attenuated the lung injury and inflammatory response to smoke exposure, as evidenced by gross, light, and electron microscopy examination. Both U75412E pretreatment and posttreatment significantly attenuated wet lung/body weight ratio (WtL/WtB). Furthermore, electron microscopy revealed that the magnitude of interstitial edema and vacuolation of type II cells were reduced by the U75412E treatment. Parallel data, by gas exchange and histopathologic analysis, suggest that the protective effect of U75412E may be related to the smoke-induced changes in capillary permeability which allows the development of alveolar interstitial edema.

The possible mechanisms of action of lazaroid in the pathogenesis of an acute smoke induced-lung injury

Lazaroids are able to integrate into cell membranes and then scavenge free radicals by their steroid moiety and the iron-chelating group [18,11,21]. Although the antioxidant properties of lazaroids have been documented in acute central nervous system trauma, cerebral ischemia and subarachnoid hemorrhage, whether and by which mechanisms lazaroids attenuate smoke-induced acute lung injury are yet to be elucidated. The present study showed that a lazaroid, U75412E, not

only had inhibition of PAM superoxide production, but substantially attenuated smoke-induced BALF TNF-α, especially PAM expression of TNF-α, in this well-characterized rabbit smoke model. In another study, mRNA levels for IL-1β, IL-6, IL-10, TNF-α, and IFN-γ were significantly decreased among intraparenchymal pulmonary mononuclear cells in mice treated with U74389F [31]. It is postulated that lazaroids may possibly be associated with the oxygen radicals-initiated processes which activate cytokine gene transcription and initiate the cytokine cascade as a result of the smoke insult.

In addition, treatment with U75412E resulted in a significant improvement in the gas exchange defect and pulmonary edema. It is reported that lung surfactant could be inactivated and/or altered both by smoke combustion products, such as nitrogen dioxide, ozone and particulate, and free radicals from activated PAM and leukocytes [11,12]. Especially, phospholipids with unsaturated fatty acids, which may be important in spreading dipalmitoylphosphatidylcholine at the air-liquid interface, are very susceptible to lipid peroxidation. Cholesterol which makes up about 8% of surfactant lipid are also subject to alteration by free radicals [12]. It is suggested that lazaroid may attenuate the smoke induced lung injury process, at least in part, by protecting alveolar surfactant from oxidation damage.

Smoke exposure can induce an acute injury to the alveolar-capillary barrier resulting in increased lung permeability progressing to ARDS by multiple cell populations [31]. Obviously, the inhibition of U75412E on free radical and TNF-α generated by PAM may be one of the major lung cells involving smoke-induced early lung injury. There is evidence that endotoxin-induced lung injury could be attenuated by another lazaroid analogue, U74389G, through a decrease in the concentration of free radicals associated with neutrophil activation [26]. Therefore, lazaroids may prove effective in those lung disease processes, including ARDS, where free-radical reactions and/or lipid peroxidation are presumed mechanisms of lung injury.

Our studies with this model have demonstrated that smoke-induced lung injury was involved in a complex series of events in the lung including the time-phase changes of PAM O_2^- production, PAM TNF-α release, and lung permeability. We found that PAM O_2^- production *in vitro* decreases at 2 hours and conversely, increases at 24 hours after cell isolation and culture in a one hour post-smoke experiment. Therefore, this investigation was designed for a comparison between pretreatment and posttreatment with U75412E in this model, due to a biphasic response of PAM O_2^- production. Our data demonstrated that the rescue mode of U75412E therapy was more advantageous than the prophylactic mode of U75412E administration in relation to gas exchange, PAM O_2^- production, and BAL TNF-α and PAM TNF-α unstimulated by LPS, and pathological evidence. Exact mechanisms of the lazaroid action due to the mode of administration (rescue-vs-prophylactic) are not known, however, the effects may be related to the lazaroid's strong scavenging ability for free radicals by their steroid moiety. Also this observation suggests, at least in part, that lipid peroxidation may not be the primary pathogenesis for the early events of injury in this smoke model. However, it may be more important for production of O_2^- to initiate lipid peroxides and/or activate cytokine gene transcription and protein expression, which could potentiate the lung injury process in the post-injury period.

The parallel evidence of gross, light and electron microscopy examination indicated that attenuating smoke-induced increases in lung permeability were associated with the pharmacological activity of U75412E. The U75412E action mechanisms in attenuating the lung permeability still need to be elucidated. We postulated that the effect of U75412E may be implicated by two mechanisms: 1) smoke exposure damages capillary interendothelial junctions to allow the development of alveolar compliance through oxidative stress and cytokine cascade; 2) smoke exposure activates the type II pneumocytes which are actively involved in pumping sodium and other electrolytes from the alveolar spaces into the interstitial areas [58,59].

Although PAM's abilities to produce O_2^- and TNF-α production and lung injury were substantially inhibited with the dose of U75412E used in this study, we can not precisely address the mechanisms of action involved, and the broader concern of lung injury. The effects of U75412E on smoke-induced lung injury could be involved in multiple mechanisms, such as the direct inhibition of lipid peroxidation and other pharmacological action mechanisms [24,27,47]. One possible key mechanism of this lazaroid's effectiveness in this smoke model may be the physicochemical interaction on the alveolar cell membrane by its iron-chelating abilities and lipophilic nature. In addition, U75412E may be associated with the oxygen radicals-initiated processes which activate nuclear transcriptional regulatory factor NF-kB, and initiate the cytokine cascade (IL-1β, TNF-α) as a result of the smoke insult. Thus, whether and how U75412E exerts its effectiveness in the model through these possible injury mechanisms requires further study.

CONCLUSIONS

1. Smoke exposure can induce an acute injury to the alveolar-capillary barrier resulting in increased lung permeability progressing to ARDS, of these process the PAM may play a central role in the early events of acute lung injury through activation of chemical mediators, such as O_2^-, TNF-α and PGI_2 production.

2. A standard dose of aerosolized U75412E (1% in saline) for 3 minutes before or after smoke exposure has been demonstrated to attenuate the acute smoke induced lung parenchyma injury by preserving gas exchange, and inhibiting PAM O_2^- and TNF-α production *in vitro*. Histopathology and cell differential data indicated that morphological alterations after smoke insult, such as inflammatory focus and interstitial edema, were also attenuated by the U75412E pretreatment.

3. It was also demonstrated that this lazaroid may be more effective as a rescue agent rather than a prophylactic agent for acute smoke induced lung injury, as evidenced by gas exchange, PAM O_2^- production, and PAM TNF-α production *in vitro* in the 2 hour experimental protocol.

4. The additional benefit from larger and/or multiple doses of U75412E remain to be experimentally demonstrated because the protection from lung injury was not complete in this study. In particular, dose- and time-effect relationships of U75412E on acute lung injury should be determined.

ACKNOWLEDGMENT

The authors thank J. Christopher Pitchford of Pharmacia-Upjohn Inc. for his assistance in this project. This research was supported by the U.S. Army Medical & Development Command, No. DAMD 17-94-J-4001 and National Institute of Health grant ES06694. The WEHI cell line 13 was a kind gift from Dr. Wagge, University of Trondheim, Norway.

The views expressed in this article are those of the authors and do not reflect the official policy or position of the Department of the Army, Department of Defense, or the U. S. Government.

REFERENCES

[1] Thompson P, Herndon DN, Abston S, Rutan T: Effect of early excision on patients with major thermal injury. *J Trauma,* 1987, **27**: 205-207.

[2] Markley, K.: Burn care: infection and smoke inhalation. *Ann. Intern. Med,* 1979, **90**: 269-270.

[3] Moritz, A. R., F. C. Henriques & R. McLean: The effects of inhaled heat on the air passages and lungs. *Am J Pathol*, 1945, **21**: 311-325.

[4] Brinkmann, B. & K. Puschel: Heat injuries to the respiratory system, *Virchows Arch. A Pathol Anat Histol*, 1978, **379**: 299-311.

[5] Bidani A, Wang CZ, Heming TA: Early effects of smoke inhalation on alveolar macrophage function. *Burns*, 1996, **22**: 101-106.

[6] Gonzalez PK, Zhuang J, Doctrow SR, Malfroy B, Benson PF, Menconi MJ, Fink MP. EUK-8, a synthetic superoxide dismutase and catalase mimetic, ameliorates acute lung injury in endotoxemic swine. *J Pharmacol Exp Ther,* 1995, **275:** 789-806.

[7] Wang W, Suzuki Y, Tanigaki T, Rank DR, Raffin TA. Effect of the NADPH oxidase inhibitor apocynin on septic lung injury in guinea pigs. *Am J Respir Crit Care Med,* 1994, **150:**1449-52.

[8] McCord JM, Gao B, Leff J, Flores SC. Neutrophil-generated free radicals: possible mechanisms of injury in adult respiratory distress syndrome. *Environ Health Perspec,* 1994, **102** (Suppl 10): 57-60.

[9] Griffin RL, Krzesicki RF, Fidler SF, Rosenbloom CL, Auchampach JA, Manning AM, Haas JV, Cammarata SK, Chin JE, Richards IM: Attenuation of oxidant-induced lung injury by 21-aminosteroids (lazaroids): correlation with the mRNA expression for E-selectin, P-selectin, ICAM-1, and VCAM-1. *Environ Health Perspect*, 1994, **102** (suppl 10): 193-200.

[10] Ozawa T, Hayakawa M, Takamura T, Sugiyama S, Suzuki K, Iwata M, Taki F, Tamita T: Biosynthesis of leukotoxin 9,10-epoxy-12-octadecenoate, by leukocytes in lung lavages of rat after exposure to hyperoxia. *Biochem Biophys Res Commun,* 1986;**134**:1071-1078.

[11] Oulton MR, Janigan DT, MacDonald JMR, Faulker GT, Scott JE: Effects of smoke inhalation on alveolar sufactant subtypes in mice. *Am J Pathol*, 1994;**145**:941-950.

[12] Krzanowski JJ: Oxidant-induced alterations of lung sufactant system. *J Fla Med Assoc*, 1994;**81**:49-51.

[13]. Kalina M, Riklis S: Alveolar type II-like cell colonies: effect of alveolar macrophages and macrophage-conditioned media. *Cell Differ*, 1988;**23**:231-236.

[14]. Panos RJ: Cytokines and alveolar type II cells. in Cytokines of the Lung, Lung Biology in Health and Disease, Vol.61, J. Kelly, ed., pp. 417-456,1993.

[15]. Whitsett JA, Clark JC, Wispe JR, Pryhuber GS: Effects of TNF-α and phorbol ester on human surfactant protein and MnSOD gene transcription *in vitro. Lung Cell Mol Physiol*, 1992;**6**:L688-L693.

[16]. Rice KL, Duane PG, Archer SL, Gilboe DP, Niewoehner DE: H_2O_2 injury causes Ca^{2+}-dependent and -independent hydrolysis of phosphatidylcholine in aveolar epithelial cells. *Lung Cell Mol Physiol*, 1992;**7**:L430-L438.

[17]. Melloni B, Lesur O, Bouhadiba T, Cantin A, Begin R: Partial characterization of the proliferation activity for fetal lung epithelial cells produced by silica-exposed alveolar macrophages. *J Leuk Biol,* 1994;**55**:574-580.

[18] Hall ED, McCall JM, Means ED. Therapeutic potential of the lazaroids (21-aminosteroids) in acute central nervous system trauma, ischemia and subarachnoid hemorrhage [Review]. *Adv Pharmacol,* 1994; **28**: 221-68.

[19] Braugher JM, Hall ED. Central nervous system trauma and stroke: I. Biochemical considerations for oxygen radicals formation and lipid peroxidation [Review]. *Free Radic Biol Med,* 1989; **6**: 289-301.

[20] Asano T, Matsui T, Takuwa Y. Lipid peroxidation, protein kinase C and cerebral vasospasm. *Crit Rev Neurosurg,* 1991; **1**: 361-379.

[21] Braughler JM, pregenzer JF, Chase RL, Duncan LA, Jacobsen EJ, McCall JM. Novel 21-amino steroids as potent inhibitors of iron-dependent lipid peroxidation. *J Biol Chem,* 1987; **262**: 10438-40.

[22] Nath KA, Balla J, Croatt AJ, Vercellotti GM. Heme protein-mediated renal injury: a protective role for 21-aminosteroids in vitro and in vivo. *Kidney Int,* 1995, **47**: 592-602.

[23] Braughler JM, Chase RL, Neff GL, Yonkers PA, Day JS, Hall ED, Sethy VH, Lahti RA: A new 21-aminosteroid antioxidation lacking glucocorticoid activity stimulates adrenocorticotropin secretion and blocks arachidonic acid release from mouse pituitary tumor (AtT-20) cells. *J Pharmacol Exp Ther*, 1988;**244**:423-427.

[24] Audus KL, Guillot FL, Braughler JM: Evidence for 21-aminosteroid association with the hydrophobic domains of brain microvessel endothelial cells. *Free Radic Bio Med,* 1991;**11**:361-371.

[25] Thomas PD, Mao GD, Rabinovitch A, Poznansky MJ: Inhibition of superoxide-generating NADPH oxidase of human neutrophis by lazaroids (21-aminosteroids and 2-methylaminochromans). *Biochem Pharmacol*,1993;**45**:241-251.

[26] Johnson D, Hurst T, Prasad K, Wilson T, Saxena A, Murphy F, Mayers I: Lazaroid pretreatment preserves gas exchange in endotoxin-treated dogs. *J Crit Care Med*, 1994, **9**: 213-223.

[27] McLaughlin GE, Frank L: Effects of the 21-aminosteroid, U74389F, on bleomycin-induced pulmonary fibrosis in rats. *Crit Care Med*, 1994, **22**: 313-319.

[28] Tanigaki T, Suzuki Y, Heimer D, Süssman HH, Ross WG, Raffin TA: Attenuation of acute injury and oxygen radical production by 21-aminosteroid, U-78518F. *J Appl Physiol*, 1993, **74**: 2155-2160.

[29] Antonini JM, van Dyke K, DiMatteo M, Reasor MJ: Attenuation of acute inflammatory effects of silica in rat lung by 21-aminosteroid, U74389G. *Inflammation*, 1995, **19**: 9-21.

[30] Aeba R, Killinger WA, Keenan RJ, Yousem SA, Hamamoto I, Hardesty RL, Griffith BP: Lazaroid U74500A as an additive to University of Wisconsin solution for pulmonary grafts in the rat transplant model. *J Thorac Cardiovasc Surg,* 1992, **104**: 1333-1339.

[31] Shenkar R, Abraham E: Effects of treatment with the 21-aminosteroid, U74389F, on pulmonary cytokine expression following hemorrhage and resuscitation. *Crit Care Med*, 1995, **23:** 132-139.

[32] Witten ML, Lantz RC, Grad R, Seidner S, Hubbard AK, Quan SF, Lemen RJ: Effect of smoke inhalation on immediate changes in lung chemical mediators. *Res Commun Chem Pathol Pharmacol,* 1991, **74**: 259-272.

[33] Brieland JK, R. G. Kunkel RG, Fantone JC: Pulmonary alveolar macrophages function during acute inflammatory lung injury. *Am Rev Respir Dis*, 1987, **135**, 1300-1306.

[34] Brieland JK, flory GM, Jones ML, Miller GR, Remick DG, Warren JS, Fantone JC: Regulation of monocyte chemoattractant protein-1 gene expression and secretion in rat pulmonary alveolar macrophages by lipopolysaccharide, tumor necrosis factor-α, and interleukin-1β. *Am. J. Respir. Cell Mol Biol*, 1995, **12**, 104-109.

[35] Kelley J.: Cytokines of the lung [Review]. *Am Rev Respir Dis*, 1990, **141**, 765-788.

[36] Xing Z, Jordana M, Kirpalani H, Driscoll KE, Schall TJ, Gauldie J: Cytokine expression by neutrophils and macrophages in vivo: endotoxin induces tumor necrosis factor-α, macrophage inflammatory protein-2, interleukin-1β, and interleukin-6 but not RANTES or transforming growth factor-β1 mRNA expression in acute lung inflammation. *Am J Respir Cell Mol Biol*, 1994, **10**, 148-153.

[37] Kojima T, Hattori K, Fujiwara T, Sasai-Takedatsu M, Kobayashi Y. Meconium-induced lung injury mediated by activation of alveolar macrophages. *Life Sci,* 1994; **54:** 1559-62.

[38] Li XY, Donaldson K, Brown D, MacNee W. The role of tumor necrosis factor in increased airspace epithelial permeability in acute lung inflammation. *Am J Respir Cell Mol Biol,* 1995; **13**: 185-95.

[39] Witten ML, Grad R, Quan SF, Sobonya RE, Hubbard AK, Lantz RC, Lentz LA, Devine LC, Lemen RJ: Piriprost pretreatment attenuates the smoke-induced increase in ^{99m}TcDTPA lung clearance. *Exp Lung Res*, 1990, **16**: 339-353.

[40] Myrvik QN, Leake ES, Fariss B: Studies on pulmonary alveolar macrophages from the normal rabbit: A technique to produce them in a high state of purity. *J Immunol*, 1961;86:128-132.

[41] Eskandari MK, Nguyen DT, Kunkel SL, Remick DG: WEHI 164 subclone 13 assay for TNF: sensitivity, specificity, and reliability. *Immunol Invest*, 1990, **19:** 69-79.

[42] Espevik T, Nissen-Meyer J: A highly sensitive cell line, WEHI 164 subclone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Methods*, 1986, **95**: 99-105.

[43] DiGregorio KA, Cilento EV, Lantz RC: Measurement of superoxide release from single pulmonary alveolar macrophages. *Am J Physiol*, 1987, **252**: C677-C683.

[44] Pinkerton KE, Crapo JD: Morphometery of the alveolar region of the lung; in Witschi HP and Brain JD (eds): Toxicology of inhaled materials. Springer-Verlag Publishers, pp.259-285, 1985.

[45] Rider ED, Witten ML, Lantz RC, Hays AM, Dizon-Co L: A free radical scavenger (Lazaroid U75412E) prevents smoke inhalation-induced changes in alveolar surfactant phospholipids. *Am Rev Respir Dis,* 1993, **147**: A363.

[46] Wang S, Lantz RC, Chen GJ, Breceda V, Rider E, Hays AM, Parliman G, Tollinger B, Rolledo RF, Kunke K, Tinajero J, Witten ML: The prophylactic effects of U75412E pretreatment in a smoke-induced lung injury rabbit model. *Pharmacol & Toxicol*, 1996, **79** (5): 231-237.

[47] Wang S, Lantz RC, Rider ED, Chen GJ, Breceda V, Hays AM, Tollinger B, Rolledo RF, Witten ML: A free radical scavenger (lazaroid U75412E) attenuates TNF-α generation in a rabbit model of smoke induced lung injury.*Respiration*, (in press).

[48] Wang S, Lantz RC, Rider ED, Chen GJ, Breceda V, Hays AM, Tollinger B, Rolledo RF, Witten ML: A 21-aminosteroid, U75412E, administered in the rescue mode attenuates superoxide production of alveolar macrophages in a model of smoke-induced lung injury. *Pharmacol Res*, (submitted).

[49] Lantz R, Chen G, Wang S, Witten M: U75412E attenuates tumor necrosis factor but not O_2- in a smoke model. *J Free Radicals Bio Med*, 1994, **9**:M:O16.

[50] Hays AM, Lantz RC, Vermeulen M, Chen G, Witten ML: U75412E pretreatment before acute smoke exposure causes a large increase in lung prostacyclin concentrations. *FASEB J,* 1993, **7**:A507.

[51] Figueroa JT, Liebler DC, Hays AM, Lantz RC, Vermeulen M, Chen G, Witten ML: U75412E pretreatment before acute smoke exposure increases BAL vitamin E levels. *FASEB J,* 1993, **7**:A408.

[52] Lantz RC, Chen GJ, Hays AM, Witten M: Alteration in alveolar macrophage function following acute smoke exposure. *FASEB J,* 1993, **7**:A367.

[53 Heppler JS, Witten ML, Lantz RC: Morphological alterations of rabbit terminal bronchiole epithelium subjected to acute smoke injury and lazaroid U75412E. *Am J Respir Crit Care Med*, 1995, **151**:A173.

[54] Lantz RC, Chen G, Wang S, Witten ML: Protective effects of inhaled lazaroid (U75412E) in a model of smoke-induced lung injury. *FASEB J*, 1995, **9**:A572.

[55] Johnson, D., T. Hurst, K. Prasad, T. Wilson, A. Saxena, F. Murphy & I. Mayers: Lazaroid pretreatment preserves gas exchange in endotoxin-treated dogs. *J Crit Care*, 1994, **9**, 213-223.

[56] Calhoun WJ, Reed HE, Moest DR, Stevens CA. Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. *Am Rev Respir Dis,* 1992, **145:** 317-25.

[57] Linares HA, Herndon DN, Traber DL: Sequence of morphologic events in experimental smoke inhalation. *J Burn Care Rehabil,* 1989, **10:**27-37.

[58] Castranova V, Jones GS, Wright JR, Colby HD, Browman L, Miles PR: Transport properties of isolated Type II alveolar epithelial cells. *Am Rev Respir Dis,* 1983, **127:** S28-33.

[59] Nord EP, Brown SE, Crandall ED: Characterization of Na^+-H^+ antiport in type II alveolar epithelial cells. *Am J Physiol,* 1987, **252**: C490-498.

[60] Van Ginkel G, Muller JM, Siemsen F, van't Veld AA, Korstanje LJ, van Zandvoot MAM, Wratten ML, Sevanian A. Impact of oxidized lipids and antioxidants such as vitamin E and lazaroids on the structure and dynamics of unsaturated membranes. *J Chem Soc Faraday Trans*, 1992; **88**:1901.

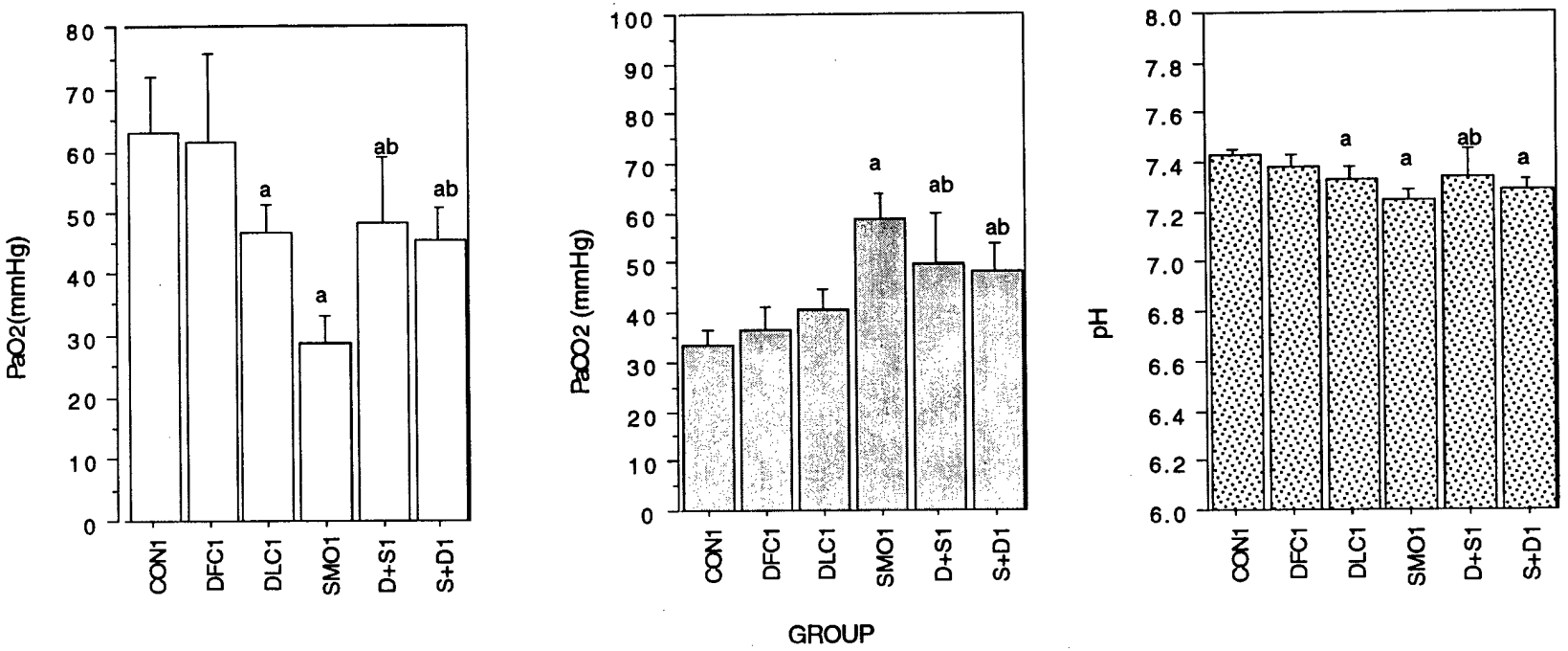

Figure 1. Comparison of the effects of U75412E pretreatment and posttreatment on gas exchange **at one hours** in a rabbit model of smoke induced lung injury. CON1: sham smoke control (n=18); DFC1: U75412E treatment immediately before sham smoke (n=17); DLC1: U75412E treatment after sham smoke (n=16); SMO1: smoke exposure (n=15); D+S1: U75412E treatment before smoke exposure (n=18); S+D1: U75412E treatment at 0.5 hour after smoke exposure (n=16). Auricular arterial blood was taken at 1 hour after smoke exposure and analyzed using a System 1620 pH/Blood Gas Analyzer and 482 Co-Oximeter. Data expressed as Mean (SEM). a $p<0.05$ compared to CON1; b $p<0.05$ compared to SMO1; c $p<0.05$ compared to D+S1.

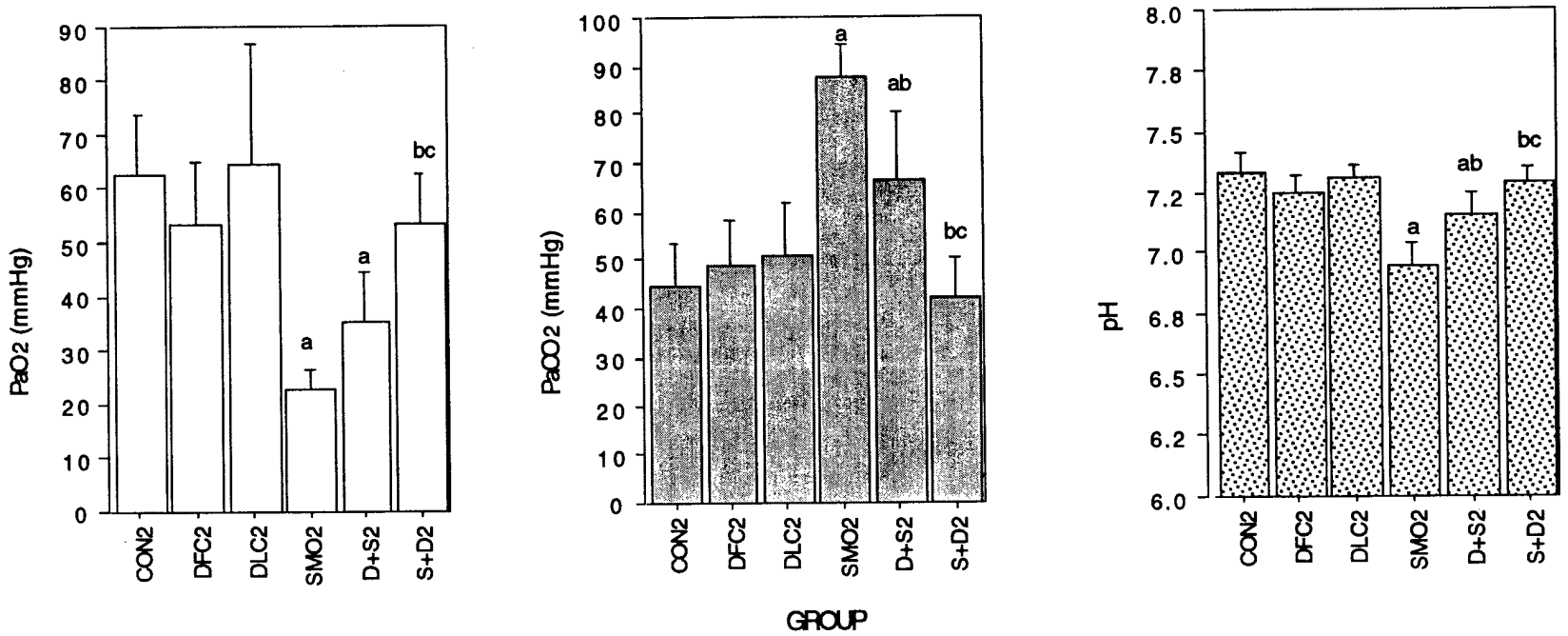

Figure 2. Comparison of the effects of U75412E pretreatment and posttreatment on gas exchange **at two hours** in a rabbit model of smoke induced lung injury. CON2: sham smoke control (n=17); DFC2: U75412E treatment immediately before sham smoke (n=14); DLC2: U75412E treatment after sham smoke (n=12); SMO2: smoke exposure (n=17); D+S2: U75412E treatment before smoke exposure (n=13); S+D2: U75412E treatment at 0.5, and 1.5 hours after smoke exposure (n=11). Auricular arterial blood was taken at 2 hours after smoke exposure and analyzed using a System 1620 pH/Blood Gas Analyzer and 482 Co-Oximeter. Data expressed as Mean (SEM). a $p<0.05$ compared to CON2; b $p<0.05$ compared to SMO2; c $p<0.05$ compared to D+S2.

Table I

Changes of cell count and differential in BALF after smoke exposure and U75412E treatment.

GROUP	CON	DFC	DLC	SMO	D+S	S+D
One hour, N=	10	8	7	9	11	8
Total cells ($X10^4$ cells/ml)	16.92±3.79	13.71±1.74	19.01±3.96	28.87±4.60[a]	17.24±4.22[b]	28.94±4.60[ac]
Cell differential (%)						
PAM	96.50±1.08	97.00±0.65	99.00±0.58	93.25±1.80	96.82±0.85[b]	94.00±1.96
Granulocyte	0.20±0.20	2.00±0.65	0.25±0.25	5.50±1.75[a]	0.91±0.44[b]	4.00±1.33[ac]
Lymphocyte	3.30±1.02	1.00±0.69	0.75±0.48	1.25±0.62	2.27±0.68	2.00±0.90
Two hours, N=	9	8	7	9	7	6
Total cells ($X10^4$ cells/ml)	34.53±5.23	15.97±2.10[a]	29.04±4.49	25.73±6.21	18.86±4.34[a]	26.97±5.37
Cell differential (%)						
PAM	98.00±0.44	97.75±0.56	97.57±0.65	98.44±0.41	98.33±0.47	97.00±0.89
Granulocyte	1.33±0.29	1.38±0.50	1.71±0.57	1.22±0.43	1.11±0.39	2.50±0.56[c]
Lymphocyte	0.67±0.37	1.00±0.26	0.71±0.18	0.33±0.23	0.56±0.24	0.50±0.34

CON: sham smoke control; DFC: U75412E treatment immediately before sham smoke; DLC: U75412E treatment after sham smoke; SMO: smoke exposure; D+S: U75412E treatment before smoke exposure; S+D: U75412E treatment after smoke exposure. Data expressed as Mean±SEM. [a] $p<0.05$ compared to CON; [b] $p<0.05$ compared to SMO; [c] $p<0.05$ compared to D+S.

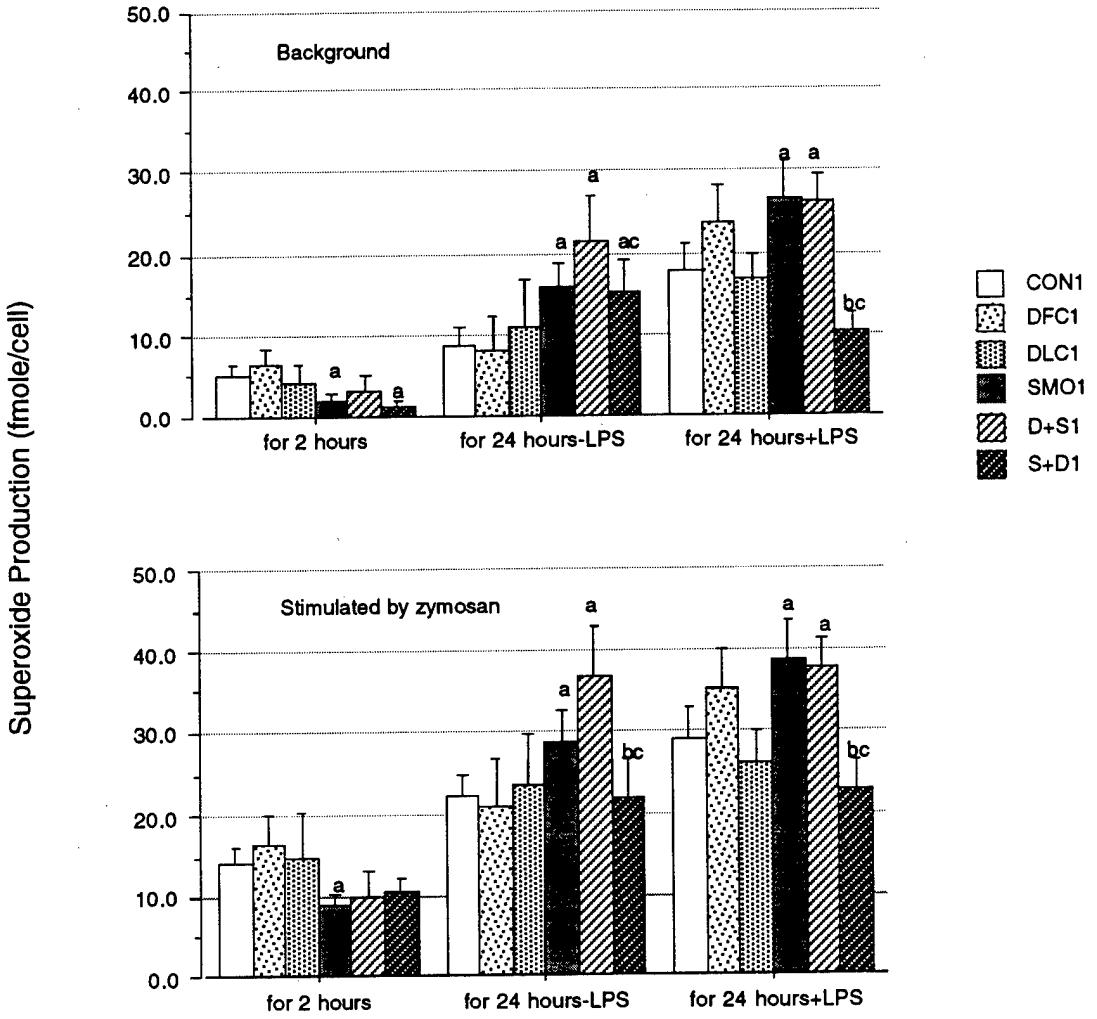

Figure 3. The effects of U75412E treatment on superoxide production of pulmonary alveolar macrophages (PAM) in a rabbit model of smoke induced lung injury. PAM were harvested at the end of one hour experiment *in vivo* and cultured for 2 and 24 hours correspondingly. CON1: sham smoke control (n=10); DFC1: U75412E treatment immediately before sham smoke (n=9); DLC1: U75412E treatment after sham smoke (n=9); SMO1: smoke exposure (n=9); D+S1: U75412E treatment before smoke exposure (n=11); S+D1: U75412E treatment at 0.5 hour after smoke exposure (n=8). Data expressed as Mean (SEM). a $p<0.05$ compared to CON1; b $p<0.05$ compared to SMO1; c $p<0.05$ compared to D+S1.

Table II

Changes of tumor necrosis factor-α (TNF-α) in BALF and PAM after smoke exposure and U75412E treatment.

GROUP	N	BALF TNF-α (pg/ml)	PAM TNF-α (pg/ml) –LPS	PAM TNF-α (pg/ml) +LPS
CON1	10	0.45 ± 0.10	2284.09 ± 470.41	3746.01 ± 1114.42
DFC1	8	1.19 ± 0.44	1435.79 ± 126.84	3929.39 ± 818.34
DLC1	9	1.37 ± 0.75	3430.76 ± 1641.77	3851.26 ± 880.46
SMO1	9	0.57 ± 0.24	7496.37 ± 1288.87[a]	3355.87 ± 1170.86
D+S1	11	0.20 ± 0.32	4046.35 ± 1742.45[b]	3787.46 ± 1176.37
S+D1	8	2,04 ± 0.02	4324.05 ± 1687.61[b]	3546.65 ± 926.49

CON1: sham smoke control; DFC1: U75412E treatment immediately before sham smoke; DLC1: U75412E treatment after sham smoke; SMO1: smoke exposure; D+S1: U75412E treatment before smoke exposure; S+D1: U75412E treatment after smoke exposure. Bronchoalveolar lavage were repeated four times with 60-ml aliquots of sterile 0.85% saline solution flushed through the tube portion of the ET with 90.2% ± 4.2 mean recovery at the end of one hour experiment *in vivo*. PAM were isolateded and cultured in RPMI medium with 25 mM HEPES in Costar 35 mm culture plates for 24 hours. Data expressed as Mean±SEM.
[a] $p<0.05$ compared to CON1; [b] $p<0.05$ compared to SMO1; Abbreviations: BALF: bronchoalveolar lavage fluid; PAM: pulmonary alveolar macrophages; LPS: lipopolysaccharide.

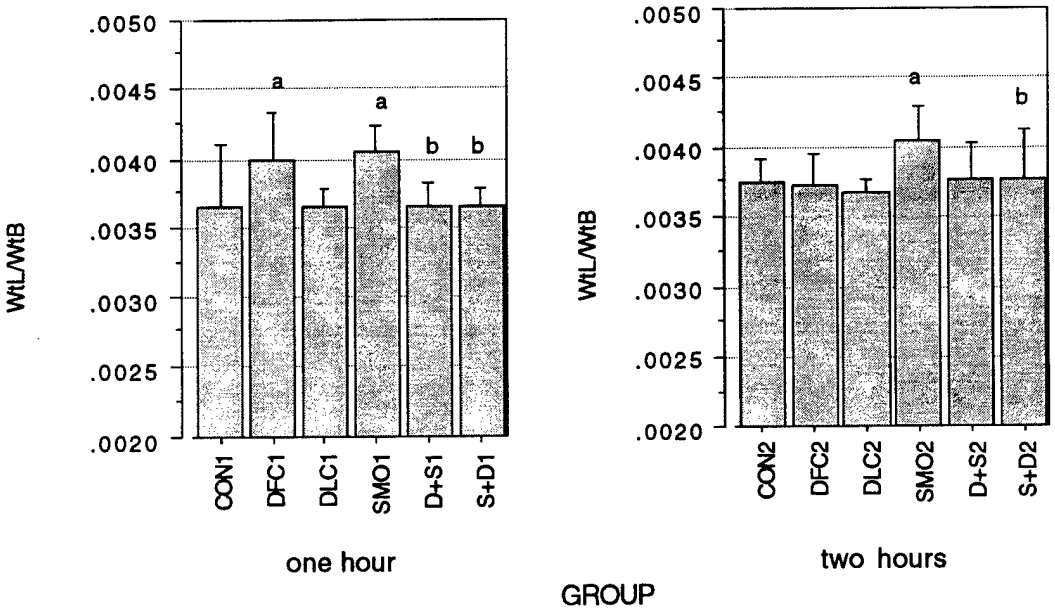

Figure 4. Comparison of the effects of U75412E pretreatment and posttreatment on wet lung/boby weight ratios (WtL/WtB) in a rabbit model of smoke induced lung injury. CON: sham smoke control; DFC: U75412E treatment immediately before sham smoke; DLC: U75412E treatment after sham smoke; SMO: smoke exposure; D+S: U75412E treatment before smoke exposure; S+D: U75412E treatment after smoke exposure. Data expressed as Mean (SEM). a $p<0.05$ compared to CON; b $p<0.05$ compared to SMO.

PUBLICATIONS GENERATED FROM U.S. ARMY PROJECT AS OF 12-17-96.

1. Wang S, Lantz RC, Chen GJ, Breceda V, Rider ED, Hays AM, Parliman G, Tollinger B, Roledo RF, Kunke K, Tinajero J, Witten ML: The prophylactic effects of U75412E pretreatment in a smoke-induced lung injury rabbit model. PHARMACOLOGY & TOXICOLOGY, 1996, 79 (5): 231-237.

2. Wang S, Lantz RC, Rider ED, Chen GJ, Breceda V, Hays AM, Tollinger B, Roledo RF, Witten ML: A free radical scavenger (lazaroid U75412E) attenuates TNF-α generation in a rabbit model of smoke induced lung injury. RESPIRATION, (in press).

3. Wang S, Lantz RC, Rider ED, Chen GJ, Breceda V, Hays AM, Tollinger B, Roledo RF, Witten ML: A 21-aminosteroid, U75412E, administered in the rescue mode attenuates superoxide production of alveolar macrophages in a model of smoke-induced lung injury. PHARMACOLOGICAL RESEARCH, (submitted).

4. Witten ML, Lantz RC, Rider ED, Wang S, Hays AM, Robledo RF, Tollinger B: U75412E treatment attentuates tumor necrosis factor-α mRNA level in a rabbit model of acute smoke induced lung injury. CRITICAL CARE MEDICINE (in preparation).

5. Rider ED, Lantz RC, Wang S, Hays AM, Robledo RF, Tollinger B, Witten ML: U75412E treatment attentuates acute smoke-induced surfactant catalysis. CRITICAL CARE MEDICINE (in preparation).

6. Wang S, Lantz RC, Rider ED, Chen GJ, Breceda V, Hays AM, Tollinger B, Roledo RF, Witten ML: Morphological alterations of rabbit terminal bronchiole and alveoli subjected to acute smoke inhalation and lazaroid U75412E. CRITICAL CARE MEDICINE (in preparation).

7. Lantz R, Chen G, Wang S, Witten M: U75412E attenuates tumor necrosis factor but not O_2- in a smoke model. JOURNAL OF FREE RADICALS IN BIOLOGY & MEDICINE, 1994, 9:M:O16.

8. Rider ED, Witten ML, Lantz RC, Hays AM, Dizon-Co L: A free radical scavenger (Lazaroid U75412E) prevents smoke inhalation-induced changes in alveolar surfactant phospholipids. AMERICAN REVIEW OF RESPIRATORY DISEASE, 1993, 147:A363.

9. Hays AM, Lantz RC, Vermeulen M, Chen G, Witten ML: U75412E pretreatment before acute smoke exposure causes a large increase in lung prostacyclin concentrations. THE FASEB JOURNAL, 1993, 7:A507.

10. Figueroa JT, Liebler DC, Hays AM, Lantz RC, Vermeulen M, Chen G, Witten ML: U75412E pretreatment before acute smoke exposure increases BAL vitamin E levels. THE FASEB JOURNAL, 1993, 7:A408.

11. Lantz RC, Chen GJ, Hays AM, Witten M: Alteration in alveolar macrophage function following acute smoke exposure. THE FASEB JOURNAL, 1993, 7:A367.

12. Heppler JS, Witten ML, Lantz RC: Morphological alterations of rabbit terminal bronchiole epithelium subjected to acute smoke injury and lazaroid U75412E. AMERICAN JOURNAL OF RESPIRATORY AND CRITICAL CARE MEDICINE, 1995, 151:A173.

13. Lantz RC, Chen G, Wang S, Witten ML: Protective effects of inhaled lazaroid (U75412E) in a model of smoke-induced lung injury. THE FASEB JOURNAL, 1995, 9:A572.

List of all personnel receiving pay, and the graduate degrees resulting from the grant support

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Raymond F. Robledo	Ph.D. Graduate		Pharmacology & Toxicology
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Elizabeth Wilson	Master's Graduate		Pharmacology & Toxicology